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Dietary β-adrenoceptor agonists have a persistent effect on nitric oxide synthesis in rat cultured smooth muscle cells

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Abstract

Several compounds including lipopolysaccharide and sympathomimetics stimulate the expression of the inducible nitric oxide synthase in vascular smooth muscle cells. We evaluated the effect of clenbuterol on nitric oxide (NO) production by vascular smooth muscle cells of the rat aorta in culture. Wistar rats were divided into three diet groups (control, clenbuterol and washout). Aortic vascular smooth muscle cells from rats from these 3 diet groups were cultured in the presence and absence of lipopolysaccharide and/or β-adrenoceptor agonists. NO release was measured by Griess reagent. Clenbuterol or salbutamol added to cells from control rats potentiated lipopolysaccharide-induced NO release. Cells from rats fed on clenbuterol, in a medium without β-adrenoceptor agonists, showed a similar potentiation, even after a 10-day washout period. The addition of β-adrenoceptor agonists to the latter cells did not increase NO production. N^G-Nitro-L-arginine decreased nitrite production in lipopolysaccharide-stimulated cells. Our results demonstrate that dietary clenbuterol has a persistent 'ex vivo' effect on lipopolysaccharide-induced NO production by cultured vascular smooth muscle cells.

Keywords: Clenbuterol; Salbutamol; Nitric oxide (NO); Smooth muscle cell

1. Introduction

Nitric oxide (NO) is a potent vasodilator synthesised from L-arginine by either the constitutive NO synthase, which is present in endothelial cells, or by an isoform of the enzyme, the inducible nitric oxide synthase, which can be induced in several cells including vascular smooth muscle cells by lipopolysaccharide and cytokines (Busse and Mülsch, 1990; Fleming et al., 1990, 1991; Rees et al., 1990; Beasley et al., 1991; Moncada et al., 1991).

Agents that increase cAMP are also able to induce NO synthase expression in vascular smooth muscle and have a synergistic action with cytokines (Koide et al., 1993; Imai et al., 1994; Scott-Burden et al., 1994).

The aim of this work is to compare the NO production in cultured aortic cells from rats fed on 3 diets (control, with clenbuterol, and after washout), in order to evaluate the persistence of the effect of dietary clenbuterol on NO production by cultured vascular smooth muscle cells.

NO production in cultured cells was measured before and after stimulation with lipopolysaccharide and when the cells were incubated in presence or in absence of different β-adrenoceptor agonists (clenbuterol and salbutamol).

2. Materials and methods

2.1. Animal diet group

WKY (Wistar Kyoto) rats were obtained from the Centre d' Elevage R.P. Janvier (Rennes, France), kept during the whole experiment period in a bioterium complying with the EU rules for animal welfare, and housed individually in metabolic cages. The animals were killed at the end of diet period by cervical dislocation, under ether anaesthesia.

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The animals were divided into three groups. The control group was fed ad libitum on a standard diet. The second group was given the same diet supplemented with clenbuterol (5 mg/kg of diet) (Rehfeldt et al., 1994) for 21 days (clenbuterol group) before the animals were killed. The third group was given clenbuterol (5 mg/kg of diet) for 21 days, but clenbuterol was removed from the diet for the next 10 days (washout period) before death (washout group).

2.2. Cell culture

The experiments were performed using vascular smooth muscle cells, isolated from thoracic aortas, obtained from weight (160–240 g) and age-matched (10–12 weeks) male WKY rats.

The cell culture technique was a modification of that described by Scott-Burden et al. (1989). After death, the thoracic aortas were removed and washed in phosphate-buffered saline (PBS) containing 200 U/ml of penicillin and streptomycin.

The thoracic aortas were opened longitudinally and shredded into fine pieces. Enzymatic dispersion was carried out with collagenase (0.3% w/v) for 2 h at 37°C followed by trypsin–EDTA (30 min) at 37°C.

After centrifugation, cells were suspended in minimal essential medium (MEM) containing Earle's salts, 20 mM glutamine, 20 mM of the buffer *N*-tris[hidroxymethyl]-methyl-2-aminoethanesulfonic acid–NaOH (TES–NaOH), 20 mM *N*-[2-hydroxyethyl] piperazine-*N′*-[2-ethane-sulfonic acid]–NaOH (HEPES–NaOH), both pH 7.4, 10% (w/v) foetal calf serum and 100 U/ml penicillin plus 100 U/ml streptomycin, and placed into tissue culture flasks (10^6 cells/flask).

After they had reached confluence, the cells were trypsinized and divided into 12-multiwell plates. When cells reached confluence the cultured medium was replaced by MEM containing 0.1% (w/v) of bovine serum albumin for 24 h, to render them quiescent.

Phenotypic characterisation was performed in the primary cultures, using monoclonal antibodies against smooth muscle α-actin (Scott-Burden et al., 1989).

2.3. Culture cell groups

In order to study unstimulated basal $NO^{\cdot}$ production, cells from each animal group were incubated without any stimulation.

To stimulate inducible NO production, cells from rats from each diet group were incubated with lipopolysaccharide from *Escherichia coli* serotype 0127:B8 (10 μg/ml) in serum-free medium for 24 h at 37°C.

After this stimulation period, the medium was changed to fresh serum-free medium. To some groups of cells test drugs—clenbuterol and salbutamol (10^{-5} and 10^{-6} M)—were added. The cells were incubated for 24 h at 37°C.

In order to confirm that the nitrite measured corresponds to nitric oxide, some stimulated cells from the control group were treated with 10 μM of N^{G}-nitro-L-arginine alone or in the presence of clenbuterol and salbutamol 10^{-5} M.

2.4. NO / Nitrite determination

Collected aliquots (500 μl) of cell supernatant were mixed with an equal volume of Griess reagent (1% sulphanilamide and 0.1% *N*-(1-naphthyl-ethylenediamine) dihydrochloride in H_3PO_4 2%). In the presence of nitrites the reagent develops a chromophore that absorbs at 530 nm (Stuehr and Marletta, 1987).

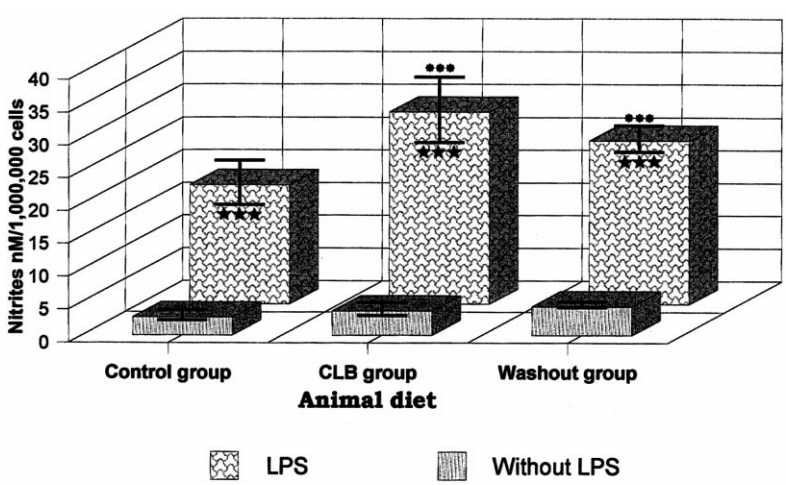

Fig. 1. Effect of animal diet on nitrite production in cultured cells, before and after lipopolysaccharide (LPS) stimulation (without sympathomimetics in the culture medium). CLB group: clenbuterol group; LPS: Lipopolysaccharide. ★★★ $P < 0.001$ Cells with and without LPS in the same animal diet group. * * * $P < 0.001$ LPS-stimulated cells vs. LPS-stimulated cells from diet control group.

The concentration of nitrite was determined by using a standard curve made with a solution of sodium nitrite in calf serum-free medium.

2.5. Statistical analysis

The nitrite production is expressed in nM/10^6 cells (mean ± S.D.). Statistical analysis was performed with Student's *t*-test for unpaired observations. *P* values less than 0.05 were considered to be statistically significant.

2.6. Material and drugs

Clenbuterol, salbutamol, N^G-nitro-L-arginine, lipopolysaccharide from Escherichia coli serotype 0127:B8, sulphanilamide, *N*-(1-naphthyl-ethylenediamine) dihydrochoride, TES and HEPES were obtained from Sigma-Aldrich Química (Madrid, Spain). Monoclonal antibodies against smooth muscle cell α-actin were from Sigma (St. Louis, USA). All cell culture reagents were from Cansera (Canada) and all plastic disposables were from Nunc (Roskilde, Denmark).

3. Results

3.1. NO production in unstimulated vascular smooth muscle cells in culture

NO production in cultured cells without drugs or lipopolysaccharide in culture medium was greater in cells from the clenbuterol group (3.63 ± 0.99, $N = 24$) than in cells from the control group (2.68 ± 0.77, $N = 26$, $P < 0.05$), and was similar to that of the washout group (4.31 ± 0.43, $N = 5$), suggesting a weak and durable stimulating effect of β-agonists on NO liberation (Fig. 1).

3.2. NO production in lipopolysaccharide-stimulated vascular smooth muscle cells in culture

NO production in unstimulated cultured cells (without lipopolysaccharide in culture medium) was much smaller than in lipopolysaccharide-stimulated cells.

In cells from the control group, NO production induced by lipopolysaccharide was not modified during the experiment (31 days) (18.17 ± 3.39, $N = 25$).

Lipopolysaccharide addition to the culture medium without β-adrenoceptor agonists induced a significantly greater NO production in cells from the clenbuterol group (29.29 ± 4.99, $N = 24$, $P < 0.001$) than in cells from the control group. In cells from the washout group, lipopolysaccharide stimulated NO production (24.94 ± 2.01, $N = 10$) was lower than that of cells from the clenbuterol group ($P < 0.01$), but was higher than that of cells from the control group ($P < 0.001$). These facts suggest that clenbuterol in the rat diet potentiated the effect of lipopolysaccharide on NO production in cultured cells even after a 10-day washout period (Fig. 1).

3.3. Incubation of lipopolysaccharide-stimulated vascular smooth muscle cells with β-adrenoceptor agonist

In cultured lipopolysaccharide-stimulated cells from the 3 groups, β-adrenoceptor agonists (clenbuterol and salbu-

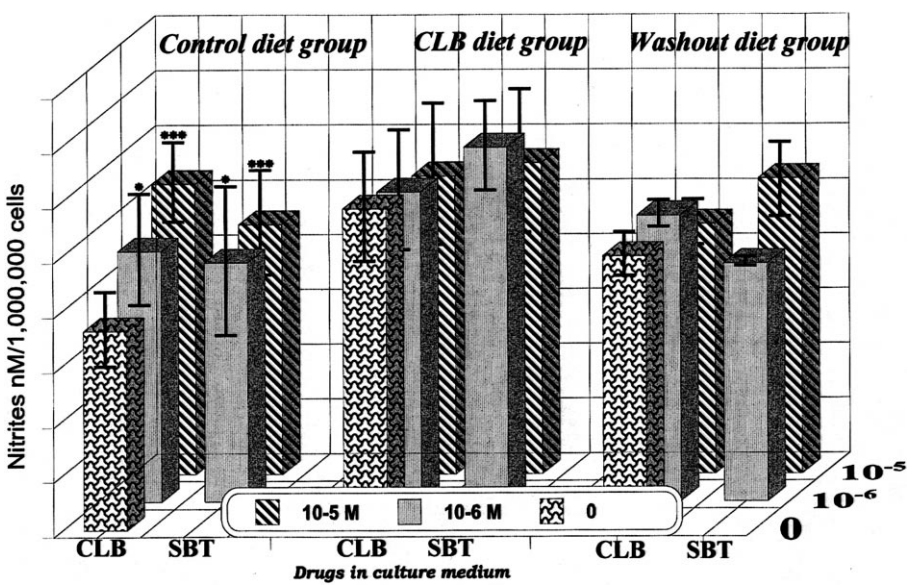

Fig. 2. Nitrite production by LPS-stimulated cultured cells from rats from the groups, in presence of sympathomimetics in the culture medium. CLB: clenbuterol; SBT: salbutamol. * $P < 0.05$; *** $P < 0.001$ LPS-stimulated cells alone vs. LPS-stimulated cells in the presence of sympathomimetics in the culture medium of cells from the control group. N.S. between LPS-stimulated cells from all diet groups incubated in presence of β-adrenoceptor in culture medium.

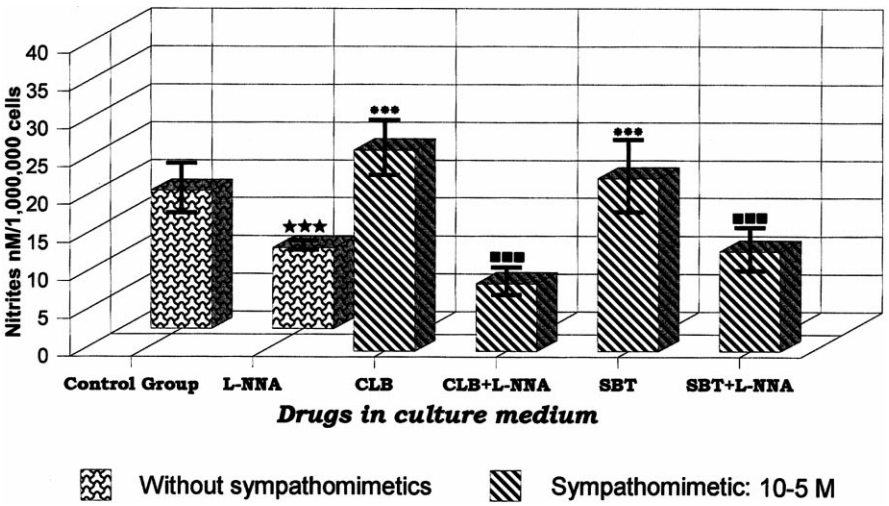

Fig. 3. Nitrite production by LPS-stimulated cultured cells from rats fed on the control diet. Effect of sympathomimetics and N^G-nitro-L-arginine (L-NNA) added to the culture medium. CLB: clenbuterol; SBT: salbutamol. $***$ $P < 0.001$ LPS-stimulated cells vs. LPS-stimulated cells in the presence of β-adrenoceptors 10^{-5} M. ★★★ $P < 0.001$ LPS-stimulated cells vs. LPS-stimulated cells incubated with L-NNA. ■ ■ ■ $P < 0.001$ LPS-stimulated cells with β-adrenoceptors vs. LPS-stimulated cells incubated with L-NNA and β-adrenoceptors.

tamol) were added to the culture medium in two concentrations, 10^{-5} and 10^{-6} M. NO production by cultured cells under these conditions was compared with that obtained after simple lipopolysaccharide stimulation.

In cells from the control group, the addition of clenbuterol and salbutamol induced a significant increase in NO production (clenbuterol 10^{-5} M: 26.52 ± 3.62, $N = 11$, $P < 0.001$; and 10^{-6} M: 22.90 ± 5.09, $N = 18$, $P < 0.05$; salbutamol 10^{-5} M: 22.80 ± 4.79, $N = 14$, $P < 0.001$; and 10^{-6} M: 21.84 ± 3.80, $N = 10$, $P < 0.05$) (Fig. 2), suggesting that the β-adrenoceptor agonist potentiated the lipopolysaccharide stimulation of inducible NO production.

In cells from the clenbuterol group, NO production elicited by lipopolysaccharide stimulation was similar in the presence or absence of clenbuterol and salbutamol in culture medium (clenbuterol 10^{-5} M: 27.20 ± 6.45, $N = 18$, NS; and 10^{-6} M: 28.29 ± 5.48, $N = 12$, NS; salbutamol 10^{-5} M: 28.39 ± 6.53, $N = 9$, NS; and 10^{-6} M: 32.34 ± 4.07, $N = 10$, NS). This suggests that the potentiation of the lipopolysaccharide effect was already present under the initial condition (without sympathomimetics in the culture medium), as a residual effect of dietary clenbuterol, and that addition of the β-adrenoceptor agonist to the medium did not induce a further increase in inducible NO production.

Lipopolysaccharide-stimulated cells from the washout group incubated in the presence or absence of clenbuterol had a similar NO production (clenbuterol 10^{-5} M: 22.79 ± 2.05, $N = 18$, NS; and 10^{-6} M: 26.10 ± 1.22, $N = 12$, NS). With salbutamol 10^{-5} M (26.95 ± 3.65, $N = 9$, NS) and 10^{-6} M (21.73 ± 0.42, $N = 4$) the results were also similar. With salbutamol 10^{-6} M the results were similar to those obtained in the cells of the control group incubated with the same concentration of the drug (21.84 ± 3.80).

3.4. Effect of N^G-nitro-L-arginine in lipopolysaccharide-stimulated cells from the control group

In lipopolysaccharide-stimulated cells from the control group nitrite production decreased from 18.17 ± 3.39 ($N = 25$) to 10.64 ± 0.6 ($N = 6$, $P < 0.001$) when the cells were exposed simultaneously to lipopolysaccharide and N^G-nitro-L-arginine (Fig. 3).

When the cells were incubated with clenbuterol (10^{-5} M) and salbutamol (10^{-5} M), in the presence of N^G-nitro-L-arginine, the nitrite production decreased from 26.52 ± 3.62 ($N = 11$) and 22.80 ± 5.09 ($N = 14$) to 8.92 ± 1.81($N = 6$, $P < 0.001$) and 13.16 ± 2.86 ($N = 5$, $P < 0.001$), respectively.

4. Discussion

β_2-adrenoceptors are located on vascular smooth muscle and mediate vasodilation. Gray and Marshal (1992) explained the vasodilation induced by β-adrenoceptor activation as being mediated by endothelial NO liberation. The infusion of the β-adrenoceptor agonist isoproterenol induces vasodilation in the forearm vessels in humans, an effect which is significantly blunted by the simultaneous administration of N^G-nitro-L-arginine (a nitric oxide synthase blocker), suggesting an important role of nitric oxide (NO) in β-adrenoceptor-mediated vasodilation (Cardillo et al., 1997).

The inducible nitric oxide synthase (enzyme that produces NO in much greater amounts than the constitutive enzyme) can be expressed in several cells including vascu-

lar smooth muscle cells. Several authors have demonstrated that vascular smooth muscle cells when stimulated by lipopolysaccharide and cytokines produce NO (Busse and Mülsch, 1990; Fleming et al., 1990, 1991; Rees et al., 1990; Beasley et al., 1991; Moncada et al., 1991). Agents that increase cAMP are also able to induce NO synthase expression in vascular smooth muscle and have a synergistic action with cytokines (Koide et al., 1993; Imai et al., 1994; Scott-Burden et al., 1994).

We observed in cells from rats fed on clenbuterol (even after 10 days washout period), without drugs or endotoxin in culture medium, a slightly greater NO liberation than that observed in cells from control rats. These results suggest a weak but persistent stimulating effect of dietary clenbuterol on NO production.

When lipopolysaccharide was added to the culture medium the NO production was significantly increased, but the greatest increase was obtained in cells from clenbuterol-fed rats, which suggests a persistent potentiation by dietary clenbuterol of the effect of lipopolysaccharide on NO production, even after the washout period. These data are also in accordance with the results described by Scott-Burden et al. (1994) for the simultaneous effect of cytokines and cAMP-increasing agents in rat aortic smooth muscle cells.

Regulation of NO production is dependent on two distinct pathways involving the induction of both NO synthase and GTP cyclohydrolase. The mechanism of synthase expression involves the action of several cytokines, among them interleukin-1β, interferon γ and TNF (tumour necrosis factor) (Bogdan et al., 1992). Additionally, NO production depends on an adequate supply of substrate (L-arginine) and an essential cofactor for inducible NOS activity, tetrahydrobiopterin. The latter production is regulated by the first enzyme of the biosynthetic pathway for pterins, namely GTP cyclohydrolase (Moncada et al., 1991; Gross and Levi, 1992; Scott-Burden et al., 1993). The cyclohydrolase enzyme is induced by direct stimulation of adenylate cyclase, resulting in elevation of cyclic AMP. Thus agents that increase the levels of intracellular cAMP regulate directly the production of tetrahydrobiopterin and the ensuing synthesis of NO (Scott-Burden et al., 1993).

In lipopolysaccharide-stimulated cells from the clenbuterol group, the addition of clenbuterol to the culture medium did not induce a further increase in NO production. This fact may suggest the possibility of a down-regulation effect on β-adrenoceptors (Re et al., 1995, 1997), which may decrease the effect of β-adrenoceptor agonists added to the culture medium. But, it seems more likely that dietary clenbuterol induced a maximum and persistent potentiation of the lipopolysaccharide effect on NO production, since the amount of NO liberated by these cells in the absence of clenbuterol in the medium was the same as that of the control group with clenbuterol in the medium. This could be explained by the increased expression of the cyclohydrolase-tetrahydrobiopterin NO synthase cofactor induced by cAMP stimulation, as demonstrated by Scott-Burden et al. (1994).

As we only measured nitrite in the culture medium, N^G-nitro-L-arginine (an inhibitor of the NO synthase) was added to some cultured cells in order to confirm that the nitrite measurement corresponds to nitric oxide liberation. As the addition of this compound decreased significantly the nitrite concentrations, we concluded that the nitrite measured corresponds to the nitric oxide produced.

The main conclusion of our work is that dietary clenbuterol has a persistent 'ex vivo' effect on NO production, especially in potentiating the effect of lipopolysaccharide on cultured cells, even after a 10-day washout period.

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